1

METHOD FOR LOWERING BOTH SEQUENCE VARIATIONS' AND INCREASE OF BASE LINE'S EFFECTS IN A DIAGNOSTIC HYBRIDISATION ASSAY, ASSAY FOR PERFORMING SUCH A METHOD AND PROBE FOR USE IN THE ASSAY

5   The present invention relates to a method for
lowering the effect of sequences variations in diagnostic
hybridisation assays that use a nucleic acid probe to detect
an amplified nucleic acid analyte. The present invention also
relates to a method for lowering the unwanted IBL (Increase of
10   Base Line) effect, which limits the use of molecular beacons
in diagnostic hybridisation assays. The invention further
relates to the assays thus obtained and to probes for use in
such assays, the diagnostic assays and probes using at least
one of these two methods for lowering undesirable consequences
15   on diagnostic results.Many diagnostic assays are based on the
amplification of a nucleic acid molecule or part thereof with
the help of primers and the detection of the amplified
material by means of a probe. Under the appropriate reaction
conditions, the primers hybridise to the analyte to be
20   detected and initiate amplification of the target sequence.
This will lead to the generation of amplicons.

          Meanwhile various amplification techniques have been
developed, such as PCR, LCR, NASBA, TMA, RCR, 3SR and SDA, and
are now well known to the person skilled in the art. Relevant
25   information on these techniques can be found in various
documents:

- PCR (Polymerase Chain Reaction), described in patents US-A-
  4,683,195, US-A-4,683,202 et US-A-4,800,159, and its derivate
  RT-PCR (Reverse Transcription PCR), to perform RNA
30   amplification disclosed particularly in EP-B-0.569.272,

- LCR (Ligase Chain Reaction), exposed in EP-A-0.201.184,

- NASBA (Nucleic Acid Sequence-Based Amplification) described in WO-A-91/02818, et

- TMA (Transcription Mediated Amplification) disclosed in patent US-A-5,399,491.

5
- RCR (Repair Chain Reaction) exposed in application WO-A-90/01069,

- 3SR (Self Sustained Sequence Replication) well explained in application WO-A-90/06995.

10   During or after amplification of the analyte or part thereof, the presence or amount of amplicons generated should be detected. This can be done with various known techniques such as separation of the sample on a gel with subsequent blotting and probing. This can only be done after the amplification is finished.

15   In a homogeneous procedure, amplification and detection occur without separating the reaction components. Amplicons are detected in the course of the amplification. Thus, the generation of amplicons can be monitored real-time and the data thus obtained can be used to determine the
20 presence or absence or the amount of the amplicon. One type of probe that is very useful in such homogeneous techniques is the molecular beacon.

Molecular beacons are single-stranded oligonucleotides having a stem-loop structure. The loop
25 portion contains the sequence complementary to the target nucleic acid (either DNA or RNA). The stem is formed due to hydridisation of the complementary sequence of the 3' end with the 5' end. The stem can be unrelated to the target and is double-stranded. One arm of the stem is labelled with a
30 fluorescent dye (fluorophore), whereas the other one is coupled to a quenching molecule. In the stem-loop state the probe does not produce fluorescence because the energy of the

fluorophore is transferred to the quenching molecule. When the
molecular beacon hybridises to the target the stem-loop
structure is lost and the quencher and fluorophore are
separated. At that stage the fluorescence emitted by the
5   fluorophore can be detected and quantified. It was recently
observed by the applicant during the development of assays
using MB's for the detection of target, that the quality of
commercially available enzymes (e.g. T7 RNA polymerase (T7))
that are normally used during the amplification process
10 differs. This led to the observation that different batches of
T7, even from the same supplier, with the same specific
activity and volume activity, had different levels of unwanted
opening of MB's (IBL effect) if these MB's consist of natural
deoxyribonucleotides. Thus a fluorescent signal appears even
15 in the absence of specific targeted sequences in the
biological sample to be tested, generating possible false
positive result.

        If skilled people could have linked the IBL effect
20 to T7 itself, our investigations showed unexpectedly that it
is not the T7 itself that generates this phenomenon, but that
it is more likely at least an unknown contaminant in the T7,
which can differ from batch to batch. In line with the
publications exposed in relation with the method for lowering
25 the effect of sequences variations in diagnostic hybridisation
assays, the man skilled in the art could have concluded to the
usefulness of a MB that consists of only 2'-O-methyl
derivatives to overcome the IBL effect. The applicant observed
unexpectedly, that it is much more efficient to introduce less
30 2'-O-methyl groups in the MB, and that substituting all
natural deoxyribonucleotides leads to non or less functional
MB's.

4

Concerning the effect of sequences variations in
diagnostic hybridisation assays, the amplicons generated in
the amplification reaction can be detected quantitatively or
5   qualitatively. In the former case the amount of amplicons
generated is quantified. In a qualitative assay only the
presence or absence of the analyte is determined.

Sequence variations (polymorphisms) in the analyte
can lead to under-quantification thereof. Also in case of
10   qualitative assays sequence variations can result in false
negatives. It is generally assumed that this is caused by
mismatches between the analyte and the primers used to amplify
the analyte or by the structure of the analyte, which causes
primers not to bind.

15   It is known that various polymorphic pathogenic
strains of viruses exist as for example for the analytes HIV,
CMV, HSV etc. These polymorphic strains differ from each other
usually by one or more nucleotides. When a primer differs from
the analyte it does not fit very well which leads to a reduced
20   amplification. When the analyte is not linear but has a
particular structure, it is less accessible by the primer,
which in turn also leads to a reduced amplification.

In addition, sequence differences between the
amplified target sequence of the analyte and the probe used
25   for detection further lower the efficiency of detection.
Analytes with polymorphisms, are thus less well detected than
analytes that match perfectly with the consensus sequence of
the probe. However, detection is usually performed at lower
temperatures than the amplification, and therefore the
30   negative effect of mismatches between probe and target is
expected to be far less than is caused by differences between
primer and analyte.

5

The probe can be optimised to fit known polymorphisms. However, in the case of unknown polymorphisms this is not possible. This is particularly a problem since new, unknown polymorphisms are continuously generated, which
5   hamper a reliable detection or quantification especially in the case of HIV.

In the research that led to the invention it was now found that in a NASBA amplification reaction of a (clinical) sample of an HIV virus with an unknown polymorphism no signal
10   or lower signals were generated in the Molecular Beacon detection system. However, surprisingly amplicons could be detected when the Molecular Beacon probe was modified such that the melting temperature of the probe with the analyte was increased. This led to the unexpected conclusion that
15   amplicons were indeed produced but not or only partially detected.

Concerning the IBL effect, the applicant also observed that this effect, due to the unwanted opening of the
20   stem of the molecular beacons by the action of contaminant(s) present within the enzymes' batch, such as the ones used during NASBA amplification (AMV-RT, T7 RNA polymerase and RNase H), can be lowered when the stem of the Molecular Beacon probes was modified in such a way that the nucleotides in the
25   stem was partly replaced by 2'-O-Methyl derivatives.

Modification of the nucleotides constituting the probes and especially the Molecular Beacon probes is a widespread technique as can be derived from the publications
30   mentioned below.

6

For example in application WO-A-00/66604, the use of
L-ribo-LNA nucleotides in constructing oligonucleotides is
exposed. L-ribo-LNAs can be used as a mean to increase
affinity and/or specificity of the probes and also to equalize
5   the affinity of different oligonucleotides for their
complementary sequences. A special form of this LNA in
combination with short probes could also be used to
discriminate between RNA and DNA probes. This can be
accomplished by replacing selected nucleosides in the
10   oligonucleotide with L-ribo-LNAs to improve diagnostic and
molecular biology procedures.

The publication of Majlessi et al. (vol. 26, no. 9,
1998, pages 2224-2229 (XP002241700)) describes linear probes
containing only 2'-O-Methyl oligonucleotides The goal of this
15   publication is limited to a comparison between 2'-O-Methyl
probes and normal 2'-deoxy probes, to show the improved Tm and
the faster hybridization kinetics of the modified probes. They
come to the conclusion that the variation of Tm values between
matched and mismatched duplexes were much greater for 2'-O-
20   Methyl probes than 2'-deoxy probes. This statement is true for
all probes length examined (8-26 nucleotides) and the
difference in Tm was most dramatic when probe lengths were 16
bases or less.

Another publication of Tsourkas et al. (vol. 30, no.
25   23, 1 December 2002, pages 5168-5174 (XP002241701)) proposes
to decrease the nuclease degradation of Molecular Beacons. For
this purpose, they protect the MB's and enhance the affinity
between probes and RNA at the same time by replacing all 2'-
deoxy nucleotides of a Molecular Beacon probe by 2'-O-Methyl
30   oligonucleotides.

In a document of Brown-Driver et al. (vol. 9, no. 2,
April 1999 (1999-04), pages 145-154 (XP009008881)), it is

7

proposed to use modified antisense oligonucleotides
complementary to HCV RNA in place of unmodified
oligonucleotides, to inhibit HCV translation. One of the
modified oligonucleotide, which is a good candidate among
5   others, is the 2'-O-Methyl oligonucleotide. As set for the two
above-mentioned publications, all the nucleotides of each
modified antisense oligonucleotide are constituted by 2'-O-
Methyl nucleotides. The consequence of this structure is the
increase of the affinity and then the strong binding between
10   the target and the modified oligonucleotides.

       In contrast to the here above mentioned prior art,
the invention shows that Molecular Beacon probes incorporating
just a few 2'-O-Methyl nucleotides per probe (between 6 and 15
of the hybridising 26 nucleotides) i.e. between 23% and 58% of
15   incorporation) are useful for RNA targets detection and even
more efficient than oligonucleotides which are totally
substituted with 2'-O-Methyl nucleotides.

       Concerning the effect of sequences variations in
20   diagnostic hybridisation assays, LNA analogues are also good
candidates to reach the final result of this invention:
Molecular Beacons, which are partially modified with LNA's,
become a very versatile tool for lowering the dependency
towards sequence variation of the target in diagnostic assays.
25   In the same way, the IBL effect due to the possible
opening of the stem-loop structure of the Molecular Beacons by
contaminant(s) present in the amplification enzymes mixture is
lowered when these Molecular Beacon probes are partially
modified with 2'-O-Methyl nucleotides. This led to the
30   unexpected conclusion that with this improved structure the
Molecular Beacon probes had a better stability and did not

8

open spontaneously in the presence of (contaminants of the amplification) enzymes.

It is therefore the object of the present invention
5   to lower the effects of sequence polymorphisms in an hybridisation assay by manipulation of the affinity between the probe and the analyte. This is achieved according to the invention by introduction into the probe of one or more nucleotides and/or nucleotide analogues that result in an
10 increase of the affinity between the analyte and the probe.

The invention thus relates to the use in a diagnostic hybridisation assay of a probe, which could be a Molecular Beacon, for lowering the effect of sequence
15 variations in a nucleic acid analyte, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe comprises one or more nucleotides
20 and/or nucleotide analogues that have an affinity increasing modification and the diagnostic assay is for assessing the amount of analyte present in the sample.
The invention also relates to the use in a diagnostic hybridisation assay of a probe, which could be a
25 Molecular Beacon, for lowering the effect of sequence variations in a nucleic acid analyte, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of
30 the probe, wherein the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, i.e. at a constant temperature of hybridisation,

9

the melting temperature of the probe with any possible analyte's polymorphism is increased compared to the melting temperature of an unmodified probe with any analyte's polymorphism and the diagnostic assay is for assessing the
5   presence of the analyte in the sample.

The invention concerns the use in a diagnostic hybridisation assay of a molecular beacon probe for lowering the IBL effect due to the possible opening of the stem of the molecular beacons by at least one contaminant present in the
10   amplification enzymes mixture, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe's stem comprises:

15   • one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, especially 2'-O-methyl nucleotides, and

• one or more unmodified nucleotides.

20   The invention also concerns the use in a diagnostic hybridisation assay of a probe for lowering:

• the effect of sequence variations in a nucleic acid analyte, and/or

• the IBL effect due to the possible opening of the stem-loop
25   structure of the molecular beacons by way of contaminated enzymes,

which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its
30   complement by means of the probe, wherein the probe's loop comprises:

10

- one or more nucleotides and/or nucleotide analogues that
  have an affinity increasing modification, and
- one or more unmodified nucleotides.
  and/or the probe's stem comprises:
5   - one or more 2'-O-methyl nucleotides that have an affinity
    increasing modification, especially 2'-O-methyl nucleotides,
    and
- one or more unmodified nucleotides.

10   Concerning the lowering of the effect of sequence
variations in a nucleic acid assay, the binding between a
probe and a target is an equilibrium between unbound target
and unbound probe on the one hand and the duplex between the
two on the other.
15   This equilibrium is described by the melting
temperature (Tm) of the duplex, which is defined herein as the
temperature at which 50% of the probe is bound to the target
nucleic acid in a duplex. Shifting the equilibrium towards the
duplex can be achieved by an increase in the melting
20   temperature. In an assay this will lead to equal
quantification of perfectly matching analytes, a better (i.e.
higher) quantification of analytes containing polymorphisms in
the sequence that is complementary to the probe and an
improved detection of very small amounts of (polymorphic)
25   analytes.
        As used herein the term "probe" is intended to
comprise a stretch of nucleotides hybridising to the target.
Preferably the hybridising part is a stretch of 10-50, more
preferably 15-35, most preferably 15-30 nucleotides.
30   The term "affinity increasing" means that the
melting temperature of a duplex between a probe comprising an

11

affinity increasing modification and an analyte is increased as compared to the melting temperature between the analyte and the unmodified probe.

The nucleotides or nucleotide analogues having a modification that increases the affinity of a probe containing them to DNA or RNA targets are preferably selected from the group consisting of 2'-O-derivatized nucleotides, locked nucleic acids (LNAs) and peptide nucleic acids (PNAs). In the case of 2'-O-derivatized nucleotides it is preferably a 2'-O-methyl-nucleotide.

The probe according to the invention is preferably a so-called molecular beacon (MB). These probes recognize their targets with higher specificity than linear probes and can easily discriminate targets that differ from one another by a single nucleotide. By the introduction of one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, and more particularly in the loop of the molecular beacon, the sensitivity to polymorphisms is lowered because the affinity of the probe to the polymorphic analyte is increased and more accurate results are obtained in the assay. Thus, the modified molecular beacon becomes a very versatile tool for lowering the dependency towards sequence variation of the target in a homogenous assay.

12

With respect to the IBL effect, the term "Molecular Beacon" probe is intended to comprise a stretch of nucleotides belonging to the sequence of the 3' end of the probe hybridising with the complementary sequence of the probe's 5'

5   end. Preferably the hybridising part is a stretch of 4-15, more preferably 5-10, most preferably 6-8 nucleotides.

The modified nucleotides can be used to synthesize the entire probe or to make a chimeric probe in which only a number of nucleotides is replaced by a modified nucleotide.

10   The amount of modifications that is minimally necessary to neutralize the mismatches is dependent on the amount of differences between the consensus sequence of an analyte and the sequence of the analyte to be detected. In general, it can be stated that the amount of modifications

15 should be such that the melting temperature of the duplex between the probe and the analyte to be detected should lie above the detection temperature, i.e. the temperature at which the detection is performed.

The amount of modified nucleotides in a probe also

20 depends on the type of modification used. The increase in Tm (the melting temperature of the probe with its target) upon introduction of a single LNA nucleotide in the probe is much higher as compared to the effect of a single 2'-O-methyl-nucleotide. In the Examples it is e.g. demonstrated that the

25 introduction of two LNA nucleotides in the probe increased the Tm with 15°C, while twelve 2'-O-methyl-nucleotides were needed to obtain the same increase in Tm.

Analytes to be detected, such as HIV, may contain so-called "hot spots" that are prone to mutations and thus

30 leading to polymorphisms. When the position of those hot spots or other positions of mismatches of the analyte and the probe are known, such as in the case of known isolates, it is

13

preferred to arrange the nucleotides or nucleotide analogues
having an affinity increasing modification around the position
of the polymorphism, preferably on conserved positions.

The present invention further relates to a
5   hybridisation assay using a nucleic acid probe to detect a
nucleic acid analyte, wherein the probe comprises one or more
nucleotides and/or nucleotide analogues that have an affinity
increasing modification. The modified nucleotides and/or
analogues thereof used in the probe are as defined above.
10

The hybridisation assays of the invention may be of
any kind in which a nucleic acid probe is used to detect a
nucleic acid analyte. Such assays may be based on the
detection of amplified analytes, such as in PCR-, TMA- or
15 NASBA-based assays. However, the probe can also be used in
arrays. The invention can be used both in quantitative and
qualitative diagnostic assays in which sequence polymorphisms
of the target influence the reliability of the assay.

Diagnostic assays that benefit from the invention
20 are for example assays for detecting viruses, bacteria and
other biomarkers as for example HIV, HBV, HCV, HSV, CMV,
Ebola, Legionella, Mycoplasma, Chlamydia, Bordetella, RSV,
MRSA, HSV, TNF-α, ER-α, as long as these diagnostic assays are
characterised in such a way that they make use of the
25 hybridisation between the analyte of interest and the modified
oligonucleotide.

The invention also relates to a probe that comprises
one or more nucleotides and/or nucleotide analogues that have
an affinity increasing modification. The probe is preferably a
30 molecular beacon.

In this application the terms ☐analyte☐, ☐amplicon☐
and ☐target☐ or ☐target sequence☐ may be used interchangeably.

14

The analyte is the original nucleic acid molecule to be
detected. The target sequence is the part of the analyte that
is amplified by means of the primers. The amplification leads
to formation of amplicons, which are the nucleic acid

5   molecules that are physically detected by hybridisation to the
probe. The sequence of the amplicons is the same or
complementary to the target sequence within the analyte.

The invention will now be described in the examples
and drawings that follow and which are not intended to limit

10   the invention in any way. Then the invention will be further
illustrated with reference to the accompanying drawings:

- Figure 1: Schematic representation of a Molecular Beacon
  allowing detection of *Legionella* bacteria, according to the
  state of the art, hereafter referred as Legionella.

15   - Figure 2: Schematic representation of an improved Molecular
  Beacon according to the present invention, that both allows
  detection of the same *Legionella* bacteria and lowers the IBL
  effect, hereafter referred as Leg-met1.

- Figure 3: Schematic representation of a Molecular Beacon
20   allowing detection of *Mycoplasma* bacteria, according to the
  state of the art, hereafter referred as Mycoplasma.

- Figure 4: Schematic representation of a first embodiment of
  improved Molecular Beacon according to the present invention
  that both allows detection of the same *Mycoplasma* bacteria
25   and lowers the IBL effect, hereafter referred as Myco-met.

- Figure 5: Schematic representation of a Molecular Beacon
  allowing detection of *Chlamydia* bacteria, according to the
  state of the art, hereafter referred as Chlamydia.

- Figure 6: Schematic representation of an improved Molecular
30   Beacon according to the present invention that both allows
  detection of the same *Chlamydia* bacteria and lowers the IBL

15

effect, hereafter referred as Chlam-met.

• Figure 7: Schematic representation of a Molecular Beacon
  allowing detection of wild type of *Enterococci* bacteria,
  according to the state of the art, hereafter referred as WT
5   Entero.

• Figure 8: Schematic representation of an improved Molecular
  Beacon according to the present invention that both allows
  detection of the same wild type of *Enterococci* bacteria and
  lowers the IBL effect, hereafter referred as WT Entero-met.

10 • Figure 9: Schematic representation of a Molecular Beacon
  allowing detection of HIV, according to the state of the
  art, incorporating 2'-*O*-methyl nucleotide neither in the
  stem nor in the loop, hereafter mentioned as Reference MB.

• Figures 10-19: Schematic representation of a series of
15   Molecular Beacons allowing detection of HIV, according to
  the state of the art, hereafter referred as MB 1 to MB 10.
  Each Molecular Beacon has a different stem that contents 2'-
  O-methyl derivatives.

• Figure 20: Bar graph representing the effect of different T7
20   enzyme batches on the IBL effect.

• Figure 21: Bar graph representing the effect of optimal stem
  structure on the varying IBL effect of different enzyme
  batches, and representing the comparative study of IBL
  effect in relation with the structure of various Molecular
25   Beacons used in an assay, i.e. unmodified Molecular Beacons
  on the one hand and modified Molecular Beacons on the other
  hand.

        In figures 20 and 21, the modified Molecular Beacons
incorporate 2'-*O*-methyl nucleotide in the stem.

30   In figures 1-8 and 10-19, the modified nucleotides
are represented by the corresponding letter, mainly G for

16

guanidine or A for adenine, in bolt, italic and underlined
manner.

General description of experimental details of the examples:

5

The examples described below use the conditions and
methods mentioned below, unless stated otherwise in the
examples.

In the experiments that use amplified RNA material,

10   this material was obtained by amplification of an RNA template
(viral lysate), which was extracted and amplified using
standard isolation and NASBA amplification conditions.

In every example the reaction conditions were kept
identical except for the variation in the type of probe

15   (Molecular Beacon) that was used.

As a model system to determine the improved subtype
reactivity, a set of well-characterized viral lysates that
represented most of the different subtypes of HIV-1 was used.

20   If an amplification reaction is being used to
generate a quantitative result, the target has been co-
amplified using a well-known amount of internal calibrator,
which has been added before the isolation. This internal
calibrator is detected using another Molecular Beacon with a

25   different colour. From the real-time amplification curves and
using specially developed software it is possible to
accurately determine the amount of material that is present.
Melting temperatures (Tm's) between target and Molecular
Beacons were determined by measuring the fluorescence

30   intensity of the mixtures as a function of the increasing
temperature. From these melting curves the Tm can be
determined. If the excess target is amplicon (RNA) the Tm is

17

referred to as Tm loop RNA. If the target is an excess of
synthetic DNA the Tm is referred to as Tm loop DNA. The latter
has been determined using synthetic DNA strand, which is
complementary towards the Molecular Beacon. In this sequence
we have introduced two C's opposite to the Inosines. In the
RNA target (amplicon) these positions contain two T's.

EXAMPLE 1

Effect of the replacing nucleotides in a probe with 2'-O-
methyl derivatives on the Tm of the duplex

The sequences of the hybridising part of three new
and one reference MB are depicted in Table 1. Also the Tm's
values of the different Molecular Beacons that have been
measured for synthetic DNA and for amplified material (RNA)
are shown in Table 1.

Table 1

Name	Tm loop DNA	Tm loop RNA	Position																								
---	---	---	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref.	58.6	48.6	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Me-1	54.7	53.8	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Me-2	58.7	62.1	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Me-7	59.4	> 65	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A

A, G = 2'-O-Me nucleotides of A and G respectively

As can be seen from the data in Table 1, the
introduction of 2'-O-methyl derivatives leads to an increased
affinity (higher Tm loop RNA) between target RNA and the
Molecular Beacon's loop. As expected, this increase is less

18

pronounced for the DNA-Molecular Beacon complex, since 2'-*O*-methyl derivatives are known to bind stronger with RNA than DNA targets.

EXAMPLE 2

Effect of replacing nucleotides in a probe with LNAs on the Tm of the duplex

In Table 2, the hybridizing sequences of two new MB's are shown together with the reference MB. The new MB's contain respectively two and three LNA building blocks. Also the Tm values for complementary DNA and for amplified material (RNA) has been measured and results are depicted in the table. From this Table it can be seen that LNA nucleotides increase the Tm of both DNA and RNA complexes. Also it can be seen that the effect on the Tm per modification is much higher as compared to the 2'-*O*-methyl nucleotides.

Table 2

Name	Tm loop DNA	Tm loop RNA	Position																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref2	45.8	57.6	A	T	C	A	A	T	G	A	G	G	A	A	G	C	T	G	C	A	G	A	A	T	G	G	G	A
LNA1	47.1	62.8	a	T	C	A	A	T	G	A	G	G	A	A	G	C	t	G	C	A	G	A	A	T	G	G	G	A
LNA2	57.5	> 65	a	T	C	A	A	T	G	A	G	G	a	A	G	C	t	G	C	A	G	A	A	T	G	G	G	A

a LNA nucleotide

EXAMPLE 3

Detection of different HIV-1 isolates with the modified Molecular Beacons

19

The HIV-1 isolates which have been selected to act as a model system to investigate the effect of five sequence polymorphisms in the target RNA sequence are shown in Table 3. These materials were available as viral lysates with a known concentration and have been used in a NASBA amplification with the Molecular Beacon's of example 1 and 2.

Table 3

Name	Position																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref. Molecular Beacon	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Subtype A	A	T	C	A	A	T	G	A	G	G	A	*A*	G	C	T	G	C	A	G	A	*A*	T	G	G	G	A
Subtype G1	A	T	*T*	A	A	T	G	A	*A*	G	A	*A*	G	C	T	G	C	A	G	A	*G*	T	G	G	G	A
Subtype N	A	T	C	A	A	T	G	A	G	G	A	*A*	G	C	*A*	G	C	A	G	A	*C*	T	G	G	G	A
Subtype O1	A	T	C	A	A	T	G	A	*T*	G	A	*A*	G	C	*A*	G	C	A	G	A	*T*	T	G	G	G	A
Subtype O3	A	T	C	A	A	T	G	A	G	G	A	*A*	G	C	*G*	G	C	A	G	A	*T*	T	G	G	G	A

The quantification results that were obtained for samples containing an equal amount of target per subtype with three of these Molecular Beacons as compared to our reference Molecular Beacon are shown in Table 4.

Table 4

subtype	Ref MB	LNA2	Me-2	Me-7
A	6.5	6.5	6.5	6.5
G	4.7	5.9	6.4	6.6
N	4.3	4.6	5.2	5.3
O1	3.3	4.9	5.4	5.9

20

O3	4.9	5.8	6.3	6.2

Since the A-subtype fits perfectly with all four Molecular Beacon's, all quantification data are normalized based on this subtype. The other subtypes have been selected since they show sequence variation with the loop-binding region of the Molecular Beacon (see Table 3). As can be seen, the highest quantifications for all five subtypes have been observed with the Me-7 Molecular Beacon derivative.

To investigate the relationship between the better quantification and the Tm loop RNA value (°C) of the different Molecular Beacon's, the latter were determined for the duplex between the Molecular Beacon derivatives and the NASBA amplicons. This yielded the results shown in Table 5.

Table 5: Tm loop RNA values (°C) for amplified material (RNA) with several MB's

subtype	ref MB	LNA2	Me-2	Me-7
A	48	65	62	65
G	45	52	58	65
N	42	55	53	60
O1	27	44	44	53
O3	40	52	54	60

As can be seen from Tables 4 and 5 a clear correlation can be observed between a higher Tm loop RNA (between the amplicon and the Molecular Beacon) and a higher quantification.

Example 4
Effect of the Stem structure on the IBL reduction

21

A series of MB's were compared that had an identical hybridising sequence towards an HIV target. The difference between the MB's was the sequence of the stem, and the content and place of 2'-O-methyl derivatives in the stem. The investigated structures are depicted in Figures 10-19. Figure 9 shows the Molecular Beacon that is used as the reference and does not contain 2'-O-methyl derivatives.

All MB's in the experiment described below were supplied to standard NASBA amplification conditions in the absence of target that could be detected with these MB's (no template reactions). The signal of the fluorophore present in the MB was measured as a function of time. From these data the IBL effect is obtained as the increase of the signal over a time period of 60 minutes. The percentage IBL is the increase as compared to the starting signal.

Table 6

MB number	IBL percentage
Reference MB	7 %
MB 1	6 %
MB 2	5 %
MB 3	3 %
MB 4	9 %
MB 5	5 %
MB 6	4 %
MB 7	5 %
MB 8	1.5 %
MB 9	0.5 %
MB 10	10 %

22

From this table it can be concluded that MB 4 and MB 10, which contain continuous stretches of 2'-O-methoxy nucleotides at either the 3' or the 5' end of the MB do not work optimally. It can also be seen from these data that the design

5 of a stem with a low IBL effect was unexpectedly low for MB 8 and MB 9.

Example 5

Effect of different T7 enzyme batches on the IBL effect

10

A MB that was designed for Enterovirus (figure 7) was compared with a MB that contained the same hybridizing sequence, but the stem structure was based on the MB 8 (see Figure 17) from the previous example. The modified MB that was designed for

15 Enterovirus is depicted in Figure 8.

The two MB's were studied in the experiment described below. They were supplied to standard NASBA amplification conditions in the absence of target that could

20 be detected with these MB's (no template reactions). Reaction conditions were kept identical for both MB's and the variations that were studied was:
    1) the effect of the introduction of the new stem in the probe and

25   2) the effect of three batches of the T7 enzyme in the Nasba reaction.   -
The three batches were obtained from the same vendor and with the same specific activity. They were different in the observed IBL effect (normal, intermediate, bad in terms of

30 IBL effect (bad being a large amount of unwanted opening of the MB)). The signal of the fluorophore present in the MB was measured as a function of time. From these data the IBL effect

23

is obtained as the increase of the signal over a time period
of 120 minutes. The percentage IBL is the increase as compared
to the starting signal.

From Figure 20, it can be seen that for all MB's
5   containing normal deoxynucleotides, the so-called "WT Entero",
the unwanted opening of the Molecular Beacons produces a
dramatic increase of the IBL effect arizing between the use of
intermediate and the use of bad T7's batches. However, the
modified MB's containing 2'-O-methoxy's in the stem (figure
10   8), show hardly any influence on the quality of the T7
batches. This clearly shows that it is the quality of the T7
(e.g. at least one contaminant of the T7 could be present)
that determines the IBL and not the T7 itself.

15

Example 6
Effect of optimal stem structure on the varying IBL effect of
different enzyme batches

A second series of MB's that were designed for
20   different targets (*Legionella* (Figure 1), *Chlamydia* (Figure 3),
Mycoplasma (Figure 5)) were compared with MB's that contained
the same hybridising sequence, but the stem structure was based
on the MB 8 (see Figure 17) from the previous example
(respectively *Legionella* (Figure 2) and *Chlamydia* (Figure 6)).
25   For the *Mycoplasma* target this was not possible, so a small
deviation on the stem structure was made as compared to the MB 8
and modified Molecular Beacons specific for this target were
obtained (see Figure 4).

30   MB's were studied in pairs (per target) in the
experiment described below. They were supplied to standard
NASBA amplification conditions in the absence of target that

24

could be detected with these MB's (no template reactions).
Reaction conditions were kept identical per set of MB's and
the variation that was studied was the introduction of the new
stem in the probe and the second variation was the use of two

5   batches of the T7 enzyme in the Nasba reaction. The first
batch (referred to as T7) was known to be good, and the second
batch (from the same vendor and with the same specific
activity) was known to generate more unwanted opening of the
Molecular Beacons producing a dramatic increase of the IBL

10   effect. The signal of the fluorophore present in the MB was
measured as a function of time. From these data, the IBL
effect is obtained as the increase of the signal over a time
period of 60 minutes. The percentage IBL is the increase as
compared to the starting signal.

15   From Figure 21, it can be seen that for all MB's
containing normal deoxynucleotides, the bad T7 results in a
higher effect of the unwanted IBL. However, the modified MB's
containing 2'-O-methoxy's in the stem showed hardly any
influence on the quality of the T7. In other words, the use of

20   methoxy derivatives in the stem structure makes the MB less
vunrable to the enzyme quality.